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(54) Title: AFFINITY-BASED PURIFICATION OF OLIGONUCLEOTIDES USING SOLUBLE MULTIMERIC OLIGONUCLEOTIDES			
(57) Abstract			
<p>The present invention provides novel compounds and methods for purifying oligonucleotides. The compounds according to the invention are multimeric oligonucleotides comprising a multimerization domain for inducing multimeric oligonucleotide aggregation, a hybridization domain that is complementary to a target oligonucleotide whose isolation is desired, and a linker domain connecting the multimerization domain and the hybridization domain. Other compounds of the invention comprise dendrimers having oligonucleotides with hybridization domains linked thereto. The methods of the invention comprise contacting the compounds of the invention with a solution containing a target oligonucleotide whose purification is desired. The target oligonucleotide hybridizes to the hybridization domain of the inventive compounds, thereby forming an aggregate. Synthetic failure sequences (N-1, N-2, etc.) and other oligonucleotides not complementary to the hybridization domain do not hybridize with the hybridization domain of the compounds and remain free in solution. Conventional size exclusion chromatography or small pore filter membranes are then used to separate the aggregate (and hence target oligonucleotide) from the other oligonucleotides. The aggregate is denatured and the target oligonucleotide separated once again by size exclusion chromatography or with a small pore filter membrane.</p>			

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AFFINITY-BASED PURIFICATION OF OLIGONUCLEOTIDES USING SOLUBLE MULTIMERIC OLIGONUCLEOTIDES

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BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to the field of purification of synthetic oligonucleotides.

Summary of the Related Art

Recent advances in the chemical synthesis of nuclease-resistant oligonucleotides (Agrawal, *Trends in Biotechnol.* 10, 152 (1992); Agrawal et al., *Opin. Biotechnol.* 6, 12 (1995)), large scale solid phase synthesis (Padmapriya et al., *Antisense Res. Dev.* 185, 4 (1994)), and purification and analytical techniques (*Methods in Molecular Biology, Vol 20, Protocols for Oligonucleotides and Analogs* (Agrawal, Ed., Humana Press, Totowa, NJ, 1993); *Methods in Molecular Biology, Vol 15, 26, Protocols for Oligonucleotide Conjugates* (Agrawal, Ed., Humana Press, Totowa, NJ, 1994)) have permitted rational design of sequence specific antisense oligonucleotides and their advancement to human clinical trials (Zhang et al., *Clin. Pharmacol. Ther.* 58, 44 (1995); Iversen et al., *Antisense Res. Dev.* 4, 43 (1994); Crooke et al., *Clin. Pharmacol. Ther.* 56, 641 (1994)). Reverse-phase HPLC, ion exchange chromatography, and gel electrophoresis are currently used for oligonucleotide purification. *Methods in Molecular Biology, Vol. 26, supra.*

Conventional methods of purification rely on such oligonucleotide characteristics as charge and hydrophobicity/hydrophilicity. Consequently, such techniques are frequently not well suited for purification of modified oligonucleotides such as methylphosphonates (no charge), 2'-O-alkyl (hydrophobic) substituted oligonucleotides, and other oligonucleotides with modifications that similarly affect

the oligonucleotides charge and hydrophobicity/hydrophilicity. Prior art techniques can also be expensive and time-consuming, particularly in large scale operations. Accordingly, new techniques for oligonucleotide purification that obviate these problems are desirable.

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SUMMARY OF THE INVENTION

The present invention provides novel multimeric oligonucleotides and their use for the purification of antisense oligonucleotides through complementary base recognition and size exclusion principles. In a particular embodiment, the invention 10 provides compounds and methods for the purification of full length synthetic oligonucleotides from a solution also containing N-1 and other failure sequences generated during synthesis. The oligonucleotides of the invention are of a unique structure that allows them to hybridize specifically with a desired, full-length oligonucleotide and concomitantly form multimer aggregates. The desired 15 oligonucleotide is thereby bound in a multimer aggregate while other undesired oligonucleotides remain in solution. The desired oligonucleotide is then separated and isolated using size-exclusion techniques.

In one aspect of the invention, the oligonucleotides according to the invention have at least three structural features: (1) a multimerization domain, (2) a 20 hybridization domain, and (3) a linker domain. The multimerization domain comprises a sequence of nucleotides that induces interoligonucleotide aggregate formation. The hybridization domain comprises a sequence of nucleotides that is complementary to the 5' end of a synthetic oligonucleotide whose purification is desired. The linker domain comprises a sequence of nucleotides or non-nucleotide 25 chemical moieties and serves both to link the hybridization domain to the

multimerization domain as well as space the hybridization domain away from the multimerization domain to allow for simultaneous aggregate formation and hybridization to the target oligonucleotide.

When contacted with a crude solution containing the desired oligonucleotide and other failure sequences generated during synthesis, the hybridization domain hybridizes to the desired ("target") oligonucleotide but not to the failure sequences, which are insufficiently complementary to hybridize to the hybridization domain under the conditions chosen. The multimerization domains concomitantly induce interoligonucleotide aggregation. The target oligonucleotide is bound in the resulting aggregate. Failure sequences remain free in solution and can be separated from the aggregates using standard size exclusion chromatographic techniques. The aggregates bearing the target oligonucleotides are then subjected to conditions that cause the target oligonucleotide to disassociate from the aggregate, and the free target oligonucleotide is isolated from the target-free aggregate by size exclusion chromatographic techniques.

In another embodiment, the present invention provides oligonucleotide dendrimers comprised of a branched chemical core structure chemically linked to oligonucleotides comprising sequences complementary to one or more target oligonucleotides. Oligonucleotide dendrimers according to the invention are used in the same way as the multimeric oligonucleotides to purify a desired oligonucleotide.

Use of the present compounds and methods results in superior purity of the desired oligonucleotide compared to prior art methods. The present invention depends on specific nucleotide base sequence recognition and not on charge or hydrophobicity/hydrophilicity. Accordingly, it is useful regardless of how the oligonucleotide is modified. A concurrent benefit of the present invention is that the

multimeric oligonucleotides are reusable. Thus, it is seen that a problem solved by the present invention is provision of compounds and methods for the improved purification of oligonucleotides.

The foregoing merely summarizes certain aspects of the present invention and 5 is not intended, nor should it be construed, to limit the invention in any way. All patents and other publications recited herein are hereby incorporated by reference in their entirety.

BRIEF DESCRIPTION OF THE DRAWINGS

10 Figure 1 displays representative oligonucleotides and dendrimers of the present invention.

Figure 2 displays the multimerization of oligonucleotide SEQ ID NO 1 (structures A and B) and the corresponding aggregate with antisense oligonucleotide SEQ ID NO 4 (structures C and D).

15 Figure 3A displays two representative oligonucleotide dendrimers of the invention with 4 and 8 branches and their hybridization with an oligonucleotide, and Fig. 3B displays a representative STARBURST™ molecule with 12 arms and its conjugate aggregate with SEQ ID NO 4.

Figure 4A displays an autoradiogram of non-denaturing polyacrylamide gel 20 showing multimeric structures formed with SEQ ID NO 1, and Fig. 4B displays an autoradiogram showing homogeneity of SEQ ID NO 4 purified with multimeric oligonucleotide SEQ ID NO 1.

Figure 5A displays an autoradiogram of a non-denaturing gel showing the mobility of crude SEQ ID NO 4 alone and as part of a complex, and Fig. 5B displays 25 an autoradiogram of a denaturing gel showing homogeneity of the bands excised from

the autoradiogram of Fig. 5A.

Figure 6 displays a schematic representation of the method of synthesizing dendrimer oligonucleotides according to the invention. The STARBURST™ dendrimer is displayed as a representative example.

5 Figure 7 displays a schematic representation of the method according to the invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides compounds and methods useful for purifying synthetic oligonucleotides. Oligonucleotides are generally synthesized in a stepwise manner, adding one nucleotide (or nucleotide dimer or trimer blocks) at a time to the 5 5' end of a nascent, solid-support-bound oligonucleotide. Because the reaction that adds each additional nucleotide has less than 100% yield, the final oligonucleotide composition is a mixture of the desired full length oligonucleotide (of "N" nucleotides in length) and failure sequences, which are shorter oligonucleotides, being one or more nucleotides shorter in length. These failure sequences are often referred to as N-10 1, N-2, etc., oligonucleotides, the number indicating how many fewer nucleotides there are as compared to the full length desired oligonucleotide. Prior art techniques for separating the full length ("target") oligonucleotide from the failure sequences (particularly the N-1 sequence) have met with limited success. The compounds and methods of the present invention provide a substantial improvement over the prior art.

15 The compounds of the invention are oligonucleotides whose structure enables them to form oligonucleotide multimer aggregates with the target oligonucleotide, but not with the failure sequences. The aggregates containing the full length oligonucleotide can then be separated from the unbound failure sequences using standard size exclusion chromatographic techniques. The methods of the invention 20 comprise the purification procedure using the oligonucleotides of the invention.

In one aspect of the invention, oligonucleotides according to the invention (herein called "multimeric oligonucleotides") have three distinct structural domains: i) a multimerization domain, ii) a flexible linker, and iii) a hybridization domain. In one embodiment of this aspect of the invention, the multimerization domain is a sequence

of 4-10 nucleotides that, when present in a longer oligonucleotide, results in the oligonucleotide aggregating into multimers, hence the name "multimerization domain." Nucleotide sequences containing four or more contiguous guanine (G) residues form multimeric structures known as G-DNA or tetraplex DNA. Sen and 5 Gilbert, *Nature* 364, 334 (1988). Consequently, in one embodiment of the invention, the multimerization domain is a short sequence of G nucleotides, generally about 4 to 10 nucleotides. The multimerization domain may contain interruptions of the G nucleotide sequence by one or two A or U nucleotides, provided that the ability of the multimerization domain to induce tetraplex formation is not defeated, which generally 10 means that it contains at least 4 contiguous G nucleotides. Preferably, from one to all of the G nucleotides is 2'-substituted with a tetraplex-stabilizing substituent. A number of such 2'-substituents are known to those skilled in the art and include, for instance, -N₃, -F, -Cl, and -OR where R is methyl, ethyl, propyl, allyl, and methoxyethoxy. Such substitutions increase the stability of the interoligonucleotide 15 tetraplex formed by the multimerization domain.

A nucleotide or non-nucleotide linker joins multimerization and hybridization domains and facilitates independent multimerization and hybridization events. The linker can be a nucleotide linker of 2 to 10 nucleotides that are not complementary to the multimerization or hybridization domains (to prevent hybridization between the 20 linker and either of these domains). The linker can also be a non-nucleotide linker of 2 to 15 constituent members such as, but not limited to, ethylene glycol, tri(ethylene glycol), tetra(ethylene glycol), penta(ethylene glycol) and hexa(ethylene glycol). Other suitable linkers are known in the art, some of which are described in U.S. Patents 5,321,131, 5,536,821, and 5,510,471.

The hybridization domain consists of a sequence of about 6 to 15 nucleotides that is complementary in the Watson-Crick sense to one of the ends of an antisense oligonucleotide whose purification is desired (e.g., for example, oligonucleotide sequence SEQ ID NO 4, *infra*). Alternatively, the hybridization domain may

5 comprise peptide nucleic acids (PNAs) having a sequence complementary to the target nucleic acid. *See* Nielson et al. (*J. Mol. Recognition* 7, 165 (1994)) for a discussion of PNAs. Although we generally recite oligonucleotides and nucleic acids in the hybridization domain as well as being targets, it will be appreciated by those skilled in the art that the hybridization domain, target, or both can be PNAs.

10 Alternatively, in this aspect of the invention, the multimeric oligonucleotides can comprise a single multimerization domain with a linker domain and corresponding hybridization domain on both the 3' and 5' end. In this embodiment the multimerization domain can be 4 to 15 nucleotides long (preferably 10-15) and is further defined as before. The linker and hybridization domains are as defined above.

15 The two linkers and the two hybridization domains of each multimeric oligonucleotide can be the same or different.

Oligonucleotides according to the invention can be modified in the base, sugar, or internucleoside linkage as desired for the particular application, e.g., to increase binding affinity. Essentially any modification is acceptable so long as it does not prevent the oligonucleotide from performing its designated function. A number of such modification are known to those skilled in the art. Agrawal and Iyer (*Current Opin. Biotech.* 6, 12 (1995)) review a number of these modifications. *See also* Uhlmann and Peyman (*Chem. Rev.* 90, 543 (1990)).

The concept underlying this aspect of the invention is schematically displayed

in Figure 2. The multimerization domains of several multimeric oligonucleotides become associated through mutual interactions (e.g., Hoogsteen base pairing in the case of oligonucleotide (G) multimerization domains). This brings several of multimeric oligonucleotides together to form an oligonucleotide aggregate (structures 5 A and B of Fig. 2). When a solution of synthetic oligonucleotides having a sequence complementary to the hybridization domain of the multimeric oligonucleotides is added, the synthetic oligonucleotides hybridize to the hybridization domain of the multimeric oligonucleotides via Watson-Crick base pairing. Conditions are selected so that N-1 and other failure sequences having fewer bases complementary to the 10 multimeric oligonucleotides' hybridization domain do not hybridize to the multimeric oligonucleotides. The appropriate conditions will depend on a number of factors, including target nucleic acid length and sequence. It is a routine matter to adjust the temperature, salt concentration (ionic strength), and pH of the buffer to obtain the desired stringency and, consequently, selectivity. The result is that the target 15 oligonucleotide becomes part of a large oligonucleotide aggregate (structures C and D in Fig. 2), whereas the synthetic oligonucleotide failure sequence remain in solution as individual (*i.e.*, unassociated) molecules. The oligonucleotide aggregates containing the target oligonucleotide are then separated from the synthetic failure sequences using standard size exclusion chromatography (e.g., sephadex or sephacryl columns) 20 under conditions selected to maintain the integrity of the aggregates. Once again, the conditions (pH, temperature, salt concentration) are application dependent but can be adjusted in a routine manner to obtain the desired result. A schematic depiction of the process is displayed in Fig. 7.

As can be appreciated from the foregoing, the linker domain serves to distance 25 the hybridization domain of the multimeric oligonucleotides from the aggregated

multimerization domain core of the oligonucleotide, making the hybridization domain appear to its binding partner (*i.e.*, the complementary target oligonucleotide) like an individual oligonucleotide free in solution. Detrimental interactions with the multimer core of the aggregate is thereby avoided, facilitating hybridization of the synthetic
5 oligonucleotide to the hybridization domain.

Oligonucleotides according to this aspect of the invention can be synthesized using standard techniques. *E.g.*, *Methods in Molecular Biology*, Vol. 20, *supra*.

Another aspect of the invention is the aggregates formed by the multimeric oligonucleotides. These aggregates generally comprise four or more multimeric
10 oligonucleotides that self-associate via the multimerization domains. *See, e.g.*, Sen and Gilbert et al.

In another embodiment, the multimerization domain is a non-nucleotide chemical moiety that contains several (about 2 to 20) amino, hydroxyl, or carboxyl functional groups (including combinations thereof) that permit attachment of
15 hybridization domain(s) (as defined above) to the multimerization domain with or without a linker. In this aspect, the present invention provides oligonucleotide dendrimers. Oligonucleotide dendrimers generally comprise two or three components: a chemical core, optionally a linker domain, and from 2 to about 16 hybridization domains. The linker domains and the hybridization domains of the
20 oligonucleotide dendrimers are the same as defined previously for the multimeric oligonucleotides. The oligonucleotides of the oligonucleotide dendrimers are linked to the chemical core either directly or through the linker domain. Two types of oligonucleotide dendrimers are displayed in Fig. 3. As can be appreciated from Fig. 3 and the purpose for which the oligonucleotide dendrimers are intended, the
25 oligonucleotides of the oligonucleotide dendrimers are preferably linked to the

chemical core (directly or through a linker) in such a manner as to make them accessible in solution for hybridization to the desired oligonucleotide.

As noted, the oligonucleotides of the oligonucleotide dendrimers have a hybridization domain that is complementary to the oligonucleotide to be purified or 5 detected. This hybridization domain can comprise the entire oligonucleotide or only a portion thereto. In either instance, the hybridization domain need only be sufficiently long so as to specifically hybridize to the oligonucleotide of interest under acceptable conditions. The only other structural constraint on the oligonucleotide dendrimers is that they do not have any modifications or other features that would interfere with 10 their principal function, *i.e.*, hybridizing with complementary oligonucleotides in solution.

Based upon the foregoing discussion and the theme depicted in Fig. 3, those skilled in the art will appreciate that a wide variety of dendrimer structures fall within the spirit and scope of the present invention, and any such structure can be used in the 15 invention so long as it conforms to the requirements set forth herein, including that it is water soluble. A number of suitable dendrimeric structures are known. *E.g.*, Newkome et al., *Aldrichemica Acta* 25, 31 (1992).

A preferred oligonucleotide dendrimer of the invention is a branched dendrimer, an example of which is shown in Figure 3A. Such dendrimers are 20 synthesized using branching CPG solid support and branching phosphoramidites. For instance, such oligonucleotide dendrimers can be synthesized on CPG (2000 or 3000 A pore size) (*see* Example 1, *infra*) attached to 1,3-didimethoxytrityl-2-glycerol. Symmetric branching phosphoramidite for use in the synthesis is commercially available (*e.g.*, Clontech, Palo Alto, CA). By this method one can control the number 25 of branches required to the range of 2-16.

Another preferred oligonucleotide dendrimer of the invention is a STARBURST™ dendrimer, as shown in Fig. 3B. STARBURST™ dendrimers are synthesized by conjugating STARBURST™ (PAMAM) dendrimer (Aldrich, Milwaukee, WI) with oligonucleotide sequences (linker and hybridization domains) 5 using the chemistry shown in Figure 6. Any generation STARBURST™ dendrimer can be used so long as the oligonucleotide dendrimer is water soluble.

Other dendrimers suitable for use in the present invention include Star PEGs and Branched PEGs (PEG = polyethylene glycol), commercially available from Shearwater Polymers, Inc. (Huntsville, Alabama). These are multi-armed PEGs made 10 by polymerization of ethylene oxide from a cross-linked divinyl benzene core. Gnanou et al., *Makromol. Chem.* **189**, 2885 (1988); Rein et al., *Acta Polymer* **44**, 225 (1993). Still other dendrimeric molecules useful in the invention can be synthesized according to the methods of Rein et al. (*Acta Polymer* **44**, 225 (1993)) and references cited therein, and Merrill (U.S. Patent No. 5,171,264), both of which disclose 15 polyethylene oxide star molecules.

Oligonucleotide dendrimers according to the invention can be synthesized with other than the STARBURST™ dendrimer using the protocol outlined in Fig. 6 with only minor modifications.

In another aspect, the invention comprises methods of separating, purifying, 20 and detecting oligonucleotides. In one embodiment of this aspect of the invention, the method comprises contacting a solution containing an oligonucleotide whose separation, purification, or detection is desired with multimeric oligonucleotides or oligonucleotide dendrimers of the invention having a hybridization domain sufficiently complementary to the desired oligonucleotide to specifically hybridize to

the desired oligonucleotide (but not to other oligonucleotides having similar sequences) under the conditions selected. After hybridization has taken place, the solution is subjected to size exclusion chromatography with an elution buffer selected to maintain the integrity of the aggregates. The elution buffer temperature, pH, and 5 salt concentration are application dependent but routine to determine. The fraction containing the multimeric oligonucleotide aggregates to which the desired oligonucleotide is bound will generally be the fastest moving component on the column. Detection is done at standard wavelengths, and the fraction containing the first component off the column is collected. The aggregate-containing fraction is 10 subject to a buffer that causes the target oligonucleotide to disassociate from the multimeric oligonucleotide or oligonucleotide dendrimer and then separated by size exclusion chromatography. As before, suitable buffer conditions are arrived at through routine adjustment of temperature, ionic strength and pH. Two fractions are collected, one containing the purified target oligonucleotide and one containing the 15 multimeric oligonucleotide or oligonucleotide dendrimer (whichever is used). The multimeric oligonucleotide or oligonucleotide dendrimer can then be reused for additional purifications.

As an alternative to size exclusion chromatography, the present methods can be conducted with a small pore filter membrane such as micricon and centricon filters 20 available from Amicon (Beverly, MA), which are particularly useful for small scale purification like those in which PCR is used. Membrane filter cartridges or pumps with suitable pore sizes can be used for large scale purification. Such filter membranes of course will have a pore size chosen to allow unbound failure sequences to pass through but not the multimeric oligonucleotide (or oligonucleotide 25 dendrimer)/target nucleic acid aggregate. In this aspect of the invention, the solution

containing the aggregate and failure sequences is filtered through the membrane. As before, the solution buffer is chosen to be of such stringency as to allow selective hybridization of the target sequence but not other oligonucleotides to the hybridization domain. The filtrate (containing the failure sequences) can be discarded if desired.

5 The material remaining on the filter, which is the aggregate, is then washed *in situ* with a buffer having a stringency that causes the target to disassociate from the oligonucleotide multimer or dendrimer oligonucleotide. The unbound target oligonucleotide will pass through the membrane. The filtrate containing the isolated oligonucleotide is collected. The membrane, on which the oligonucleotide multimer

10 or dendrimer oligonucleotide is, can be washed and the oligonucleotide multimer or dendrimer oligonucleotide collected and used again.

The present compounds and methods can also be used to isolated segments from a genome. In this aspect of the invention the hybridization domain of the multimeric oligonucleotide or oligonucleotide dendrimer is complementary in the 15 Watson-Crick, Hoogsteen, reverse Watson-Crick, or reverse Hoogsteen sense. The genome or portion thereof containing the segment of interest (the target segment) is subject to restriction endonucleases and the resulting hydrolysate subject to the methods of the invention. If the target segment is purine rich, the hybridization domain of the multimeric oligonucleotide or oligonucleotide dendrimer may be 20 complementary in the Hoogsteen or reverse Hoogsteen sense to the purine rich segment so that the double stranded target segment hybridizes to the hybridization domain to form a triplex. Alternatively, where the target segment is a mixed sequence of purine and pyrimidines, it is preferably denatured first and the hybridization domain is complementary in the Watson-Crick sense to one of the strands.

It will be appreciated from the foregoing that the compounds and methods of the invention provide several advantages over prior art techniques. The present methods can be used to purify any molecule capable of specific hybridization based on complementary nucleotide base sequence recognition through Watson-Crick, reverse 5 Watson-Crick, Hoogsteen, or reverse Hoogsteen hydrogen bonding, including but not limited to nucleic acids and PNAs. Most notably, the methods of the invention do not rely on charge, hydrophobicity, or hydrophilicity and, therefore, are suitable regardless of how the target oligonucleotide (or PNA) is modified. An additional 10 advantage of the methods of the invention is that the multimeric oligonucleotide can be used repeatedly for a number of purifications, thereby reducing the cost. The results presented herein demonstrate that the multimeric structures can be used to purify antisense oligonucleotides as shown in Fig. 7.

From the foregoing, those skilled in the art will appreciate that the compounds and methods of the invention are not limited to isolating a single synthetic 15 oligonucleotide from a mixture of failure sequences, but can be used to isolate a desired oligonucleotide from any composition in solution. Furthermore, by using compounds of the invention with two or more different hybridization domains or mixtures of the compounds of the invention in which each compound has one of several different hybridization domains, more than one target oligonucleotide can be 20 separated at a time. In this embodiment, multimeric oligonucleotides or asymmetric branching dendrimers (commercially available from, for example, Clontech (Palo Alto, CA)) are used.

The following examples are provided for illustrative purposes only and are not intended, nor should they be construed, to limit the invention in any way. Those 25 skilled in the art will appreciate that variations can be made without violating the

scope or spirit of the invention.

EXAMPLES

Figure 1 displays the oligonucleotide sequences and aggregates used in this study. The three boxes (from left to right) in each of the first three structures represent multimeric, linker, and hybridization domains, respectively, as described herein. In dendrimer 2, the number of branches is equal to $2n$, and where n is 2-4. In dendrimer 3, the number of branches is equal to n' , and where n' is 2-16 ($n'=12$ is shown in Fig. 3). Oligonucleotide SEQ ID NO 4 is the antisense oligonucleotide whose purification is undertaken, and the underlined portion is complementary to the hybridization domains of oligonucleotide SEQ ID NO 1 and dendrimers 2 and 3.

Example 1

Synthetic Methods

15 Oligonucleotide Synthesis

Oligonucleotides were synthesized on a Milligen 8700 DNA synthesizer (Bedford, MA) using phosphoramidite chemistry. P-cyanoethyl-N, N-diisopropyl phosphoramidites were purchased from Millipore for DNA synthesis. Oligonucleotide SEQ ID NO 2 was synthesized on CPG (2000 or 3000 Å pore size) 20 attached to 1,3-didimethoxytrityl-2-glycerol. Symmetric branching phosphoramidite was obtained from Clontech (Palo Alto, CA). After attaching the required number of branches on the synthesizer, oligonucleotide synthesis was continued with normal P-cyanoethyl-N,N-diisopropyl phosphoramidites as described above.

After synthesis, oligonucleotides SEQ ID NOs 1 and 2 were deprotected, 25 purified on reverse phase HPLC (C_{18}), detritylated, and desalted using C_{18} Sep-pack

cartridges (Waters, Milford, MA). Oligonucleotide SEQ ID NO 4 was deprotected and used as is without purification.

Synthesis of Branching Dendrimer

5 We synthesized 1,3- di-dimethoxytrityl-2-hydroxy-glycerol by treating glycerol with 2 equivalents of 1,1'-dimethoxytrityl chloride (DMTCl) in pyridine for 24 hrs at room temperature and for additional 7 hrs at 50°C to allow completion of the second hydroxy group with DMTCl. The di-DMT-2-hydroxy glycerol was purified by flash column chromatography on silica gel 60 (<230 mesh ASTM, Merck, 10 Darmstadt, Germany) by eluting with a mixture of hexane:methylene chloride:triethylamine (20:4:1). Yield of the purified white diDMT product was 94%.

The diDMT product obtained was loaded on to long chain alkylamido propionic acid controlled-pore glass (CPG) supports (CPG, Inc., Lincoln Port) having pore sizes of 2000 and 3000 Å as reported (Damha et al., *Nucleic Acids Res.* **18**, 3813 15 (1990)) to yield 1,3- diDMT-2-hydroxy-glycerol on CPG. The loading efficiencies were 13.8 µmoles/gm (2000 Å) and 9.3 µmoles/gm (3000 Å).

Conjugation of Oligonucleotide to STARBURST™ Dendrimer

The 3'-DMT-5'-P-cyanoethyl-N,N-diisopropyl phosphoramidites and 20 5'-monomer attached CPG solid support were obtained from Glen Research Laboratories (Sterling, VA) or Chemgenes (Waltham, MA). Dendrimer 5 (Fig. 6) was synthesized on a 5'-monomer attached CPG using 3'-DMT- 5'-phosphoramidite monomers with longer detritylation coupling times and in trityl-off mode. Then the CPG-attached oligonucleotide with free 3'-OH was activated with bis(p-nitrophenyl) 25 carbonate in anhydrous 1,4-dioxane with triethylamine as catalyst (3 drops) for 1 hr to

obtain active carbonate dendrimer 6 (Fig. 6) as described by Habus et al. (*Bioorganic and Med. Chem. Lett.* 4, 1065 (1994)) and Habus et al. (*Bioconjugate Chem.* 6, 327 (1995)). The active oligonucleotide carbonate was then successively washed with anhydrous 1,4-dioxane and acetonitrile and dried in vacuum. The activated 5 oligonucleotide was taken in anhydrous pyridine/methanol and added to STARBURST™ (PAMAM) dendrimer, generation 2 (Mol. Wt. 3,256) (Aldrich Chemical Co. Milwaukee) and shaken in a mechanical shaker for 2 hrs at room temperature to give dendrimer 7 (Fig. 6). Then the product was washed with pyridine and methanol. Dendrimer 3 (Fig. 1) was cleaved from CPG and deprotected as 10 described above. Then the aggregate was purified on polyacrylamide gel.

Example 2

Purification With Multimeric Oligonucleotides

Crude oligonucleotide SEQ ID NO 4 was labeled at the 5'-end with ^{32}P using
15 T4-polynucleotide kinase (Promega, Madison, WI), and γ - ^{32}P -dATP (Sambrook et al.,
Molecular Cloning (Cold Spring Harbor Press, Cold Spring Harbor, New York,
1989.)). Tetraplex (multimeric structure) formation was confirmed on gel using
5'-end ^{32}P labeled oligonucleotide SEQ ID NO 1 in 200 mM potassium acetate. For
purification experiments, the required amount of labeled, cold oligonucleotide SEQ ID
20 NO 4 was mixed with oligonucleotides SEQ ID NO 1 in 10 mM sodium dihydrogen
phosphate (pH 7.6) and 100 mM sodium chloride and incubated for 30-60 min. Then
the samples were loaded on 20% non-denaturing polyacrylamide gel and
electrophoresed. The slow moving band was excised from the gel and eluted with 0.5
mM sodium acetate (pH 7.2) overnight and ethanol precipitated. The DNA sample
25 was then mixed with formamide gel loading buffer and analyzed on a denaturing 7 M

urea polyacrylamide gel.

Figure 4A is an autoradiogram of non-denaturing polyacrylamide gel showing multimeric structures formed with oligonucleotide SEQ ID NO 1. Lanes 1 and 2 are markers of 22-base long single- and double-stranded DNAs, respectively. Lanes 3-6 5 contain oligonucleotide SEQ ID NO 1 incubated in 100 mM NaOAc, pH 7.6 (lane 3), 200 mM NaOAc, pH 7.6 (lane 4), 100 mM KOAc, pH 7.6 (lane 5), and 200 mM KOAc, pH 7.6 (lane 6). The figure shows that SEQ ID NO 1 forms multimeric structure (tetraplex) as expected.

Figure 4B is an autoradiogram showing homogeneity of SEQ ID NO 4 10 purified with multimeric oligonucleotide SEQ ID NO 1. Lanes 5 and 6 are crude SEQ ID NO 4 before and after treating with SEQ ID NO 1. Lane 1 is purified SEQ ID NO 4 by standard methods. Lanes 2-4 are SEQ ID NO 4 prepurified by conventional techniques and subsequently treated with SEQ ID NO 1 as in the case of crude SEQ ID NO 4. This figure shows that the isolated fraction contains only pure antisense 15 oligonucleotide SEQ ID NO 1 (lanes 2, 3, 4, and 6) (compare with lanes 1 and 5, which contain purified and crude oligonucleotide SEQ ID NO 4, respectively).

Example 3

Purification With Oligonucleotides Dendrimers

20 We used oligonucleotide dendrimers shown in Figure 3A to demonstrate their use for purification of antisense oligonucleotide SEQ ID NO 4. Dendrimer 2 (Fig. 1) was used for purification in the same manner as SEQ ID NO 1, as described in Example 2.

Figure 5A is an autoradiogram of a non-denaturing gel showing the mobility 25 of the complexes of crude SEQ ID NO 4 with 2 and 4 branched dendrimer 2 (Fig. 1)

(lanes 3 and 4, respectively). Lane 1 is crude SEQ ID NO 4 alone. Lane 2 is crude SEQ ID NO 4 with a 25 base long complementary sequence. Note that the failure sequences are seen as a diffuse band below the dark band in lane 1, not seen in lane 2, but present in lanes 3 and 4. Appropriate bands were cut, eluted, and run on a 5 denaturing polyacrylamide gel to examine the homogeneity of oligonucleotide SEQ ID NO 4. Figure 5B is an autoradiogram of a denaturing gel showing homogeneity of the bands excised from lanes 2 (lane 1), 3 (lane 2) and 4 (lane 3) of the gel shown in Fig. 5A. Lanes 2 and 3 of Fig. 5B suggest that the oligonucleotide is pure and the failure sequences are absent. Similar results obtained with the oligonucleotide 10 dendrimer shown in Fig. 3B.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

5

(i) APPLICANT: Agrawal Ph.D., Sudhir
Habus Ph.D., Ivan
Kandimalla Ph.D., Ekambar R.

10 (ii) TITLE OF INVENTION: Multimeric Oligonucleotides
and Their
Use for Oligonucleotide Purification

15 (iii) NUMBER OF SEQUENCES: 4

15

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Banner & Allegretti, Ltd.
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(C) CITY: Chicago
20 (D) STATE: IL
(E) COUNTRY: USA
(F) ZIP: 60606

(v) COMPUTER READABLE FORM:

25 (A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version
#1.30

30

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

35

(viii) ATTORNEY/AGENT INFORMATION:

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(B) REGISTRATION NUMBER: 37,142
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40

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45

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TGCGAGAGTT

TTTTTTGGGG

GG

22

15

(2) INFORMATION FOR SEQ ID NO:2:

20 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGTGCGAGAG

TTT

13

35

(2) INFORMATION FOR SEQ ID NO:3:

40 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

5 TTTTTTTTGG GGGG
14

(2) INFORMATION FOR SEQ ID NO:4:

10 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

20 (iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

25 CTCCTCGCACC CATCTCTCTC

We claim:

1. A multimeric oligonucleotide comprising a multimerization domain, which comprises a sequence of chemical moieties capable of inducing interoligonucleotide aggregation, a hybridization domain, which comprises a sequence of nucleotides that 5 is complementary to an oligonucleotide or peptide nucleic acid whose purification is desired, and a linker domain, which comprises a sequence of chemical moieties that physically connects the hybridization domain to the multimerization domain.
2. The oligonucleotide according to claim 1 wherein the multimerization domain 10 comprises a sequence of 4 or more contiguous G nucleotides.
3. The oligonucleotide according to claim 2 wherein one or more of the G nucleotides has a tetraplex-stabilizing 2'-substituent.
- 15 4. The oligonucleotide according to claim 3 wherein the hybridization domain comprises a sequence of from about 6 to about 15 nucleotides.
5. The oligonucleotide according to claim 4 wherein the linker domain comprises from about 2 to about 10 nucleotides.
- 20 6. The oligonucleotide according to claim 4 wherein the linker domain comprises from about 2 to about 15 non-nucleotide chemical moieties selected from the group consisting of ethylene glycol, tri(ethylene glycol), tetra(ethylene glycol), penta(ethylene glycol), and hexa(ethylene glycol).
- 25 7. An oligonucleotide dendrimer comprising a chemical core structure and from 1 to about 16 oligonucleotides that are complementary to a target oligonucleotide whose

purification is desired.

8. The oligonucleotide dendrimer according to claim 7 wherein the chemical core structure is a branched dendrimer.

5 9. The oligonucleotide dendrimer according to claim 7 wherein the chemical core structure is the STARBURST™ dendrimer.

10. The oligonucleotide dendrimer according to claim 8 wherein the oligonucleotides comprise a sequence of about 6 to about 15 nucleotides.

10 11. The oligonucleotide dendrimer according to claim 8 further comprising a linker region.

12. The oligonucleotide dendrimer according to claim 9 wherein the linker region 15 comprises a sequence of from about 2 to about 10 nucleotides.

13. The oligonucleotide dendrimer according to claim 9 wherein the linker domain comprises from about 2 to about 15 non-nucleotide chemical moieties selected from the group consisting of ethylene glycol, tri(ethylene glycol), tetra(ethylene glycol), 20 penta(ethylene glycol), and hexa(ethylene glycol).

14. A method of purifying a target oligonucleotide or PNA comprising contacting the target with a multimeric oligonucleotide according to claim 1 under conditions in which the hybridization domain specifically hybridizes to the target to form a 25 multimeric oligonucleotide/target oligonucleotide aggregate, separating the aggregate from the mixture of oligonucleotides, disassociating the target oligonucleotide from the aggregate, and separating the target oligonucleotide from multimeric

oligonucleotide.

15. A method of purifying a target oligonucleotide or PNA comprising contacting the target with a multimeric oligonucleotide according to claim 2 under conditions in which the hybridization domain specifically hybridizes to the target to form a multimeric oligonucleotide/target oligonucleotide aggregate, separating the aggregate from the mixture of oligonucleotides, disassociating the target oligonucleotide from the aggregate, and separating the target oligonucleotide from multimeric oligonucleotide.
- 10 16. A method of purifying a target oligonucleotide or PNA comprising contacting the target with a multimeric oligonucleotide according to claim 3 under conditions in which the hybridization domain specifically hybridizes to the target to form a multimeric oligonucleotide/target oligonucleotide aggregate, separating the aggregate from the mixture of oligonucleotides, disassociating the target oligonucleotide from the aggregate, and separating the target oligonucleotide from multimeric oligonucleotide.
- 15 17. A method of purifying a target oligonucleotide or PNA comprising contacting the target with a multimeric oligonucleotide according to claim 4 under conditions in which the hybridization domain specifically hybridizes to the target to form a multimeric oligonucleotide/target oligonucleotide aggregate, separating the aggregate from the mixture of oligonucleotides, disassociating the target oligonucleotide from the aggregate, and separating the target oligonucleotide from multimeric oligonucleotide.

18. A method of purifying a target oligonucleotide or PNA comprising contacting the target with a multimeric oligonucleotide according to claim 5 under conditions in which the hybridization domain specifically hybridizes to the target to form a multimeric oligonucleotide/target oligonucleotide aggregate, separating the aggregate from the mixture of oligonucleotides, disassociating the target oligonucleotide from the aggregate, and separating the target oligonucleotide from multimeric oligonucleotide.

5

19. A method of purifying a target oligonucleotide or PNA comprising contacting the target with a multimeric oligonucleotide according to claim 6 under conditions in which the hybridization domain specifically hybridizes to the target to form a multimeric oligonucleotide/target oligonucleotide aggregate, separating the aggregate from the mixture of oligonucleotides, disassociating the target oligonucleotide from the aggregate, and separating the target oligonucleotide from multimeric oligonucleotide.

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20. A method of purifying a target oligonucleotide or PNA comprising contacting the target with a multimeric oligonucleotide according to claim 7 under conditions in which the hybridization domain specifically hybridizes to the target to form a multimeric oligonucleotide/target oligonucleotide aggregate, separating the aggregate from the mixture of oligonucleotides, disassociating the target oligonucleotide from the aggregate, and separating the target oligonucleotide from multimeric oligonucleotide.

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21. A method of purifying a target oligonucleotide or PNA comprising contacting the target with a multimeric oligonucleotide according to claim 8 under conditions in

which the hybridization domain specifically hybridizes to the target to form a multimeric oligonucleotide/target oligonucleotide aggregate, separating the aggregate from the mixture of oligonucleotides, disassociating the target oligonucleotide from the aggregate, and separating the target oligonucleotide from multimeric 5 oligonucleotide.

22. A method of purifying a target oligonucleotide or PNA comprising contacting the target with a multimeric oligonucleotide according to claim 9 under conditions in which the hybridization domain specifically hybridizes to the target to form a multimeric oligonucleotide/target oligonucleotide aggregate, separating the aggregate 10 from the mixture of oligonucleotides, disassociating the target oligonucleotide from the aggregate, and separating the target oligonucleotide from multimeric oligonucleotide.

23. A method of purifying a target oligonucleotide or PNA comprising contacting 15 the target with a multimeric oligonucleotide according to claim 10 under conditions in which the hybridization domain specifically hybridizes to the target to form a multimeric oligonucleotide/target oligonucleotide aggregate, separating the aggregate from the mixture of oligonucleotides, disassociating the target oligonucleotide from the aggregate, and separating the target oligonucleotide from multimeric 20 oligonucleotide.

24. A method of purifying a target oligonucleotide or PNA comprising contacting the target with a multimeric oligonucleotide according to claim 11 under conditions in which the hybridization domain specifically hybridizes to the target to form a 25 multimeric oligonucleotide/target oligonucleotide aggregate, separating the aggregate from the mixture of oligonucleotides, disassociating the target oligonucleotide from

the aggregate, and separating the target oligonucleotide from multimeric oligonucleotide.

25. A method of purifying a target oligonucleotide or PNA comprising contacting
5 the target with a multimeric oligonucleotide according to claim 12 under conditions in
which the hybridization domain specifically hybridizes to the target to form a
multimeric oligonucleotide/target oligonucleotide aggregate, separating the aggregate
from the mixture of oligonucleotides, disassociating the target oligonucleotide from
the aggregate, and separating the target oligonucleotide from multimeric
10 oligonucleotide.

26. A method of purifying a target oligonucleotide or PNA comprising contacting
the target with a multimeric oligonucleotide according to claim 13 under conditions in
which the hybridization domain specifically hybridizes to the target to form a
15 multimeric oligonucleotide/target oligonucleotide aggregate, separating the aggregate
from the mixture of oligonucleotides, disassociating the target oligonucleotide from
the aggregate, and separating the target oligonucleotide from multimeric
oligonucleotide.

20 27. An aggregate comprising four or more oligonucleotides according to claim 1.

28. An aggregate comprising four or more oligonucleotides according to claim 2.

29. An aggregate comprising four or more oligonucleotides according to claim 3.

25 30. An aggregate comprising four or more oligonucleotides according to claim 4.

31. An aggregate comprising four or more oligonucleotides according to claim 5.

32. An aggregate comprising four or more oligonucleotides according to claim 6.
33. A multimeric oligonucleotide comprising a multimerization domain, which
5 comprises a sequence of chemical moieties capable of inducing interoligonucleotide aggregation, two hybridization domains, each of which comprise a sequence of nucleotides that is complementary to an oligonucleotide or peptide nucleic acid whose purification is desired, and two linker domains, each of which comprises a sequence of chemical moieties that physically connects one of the hybridization domains to the
10 multimerization domain, wherein the linker domains are the same or different and the hybridization domains are the same or different.
34. A method of purifying a target oligonucleotide or PNA comprising contacting the target with a multimeric oligonucleotide according to claim 33 under conditions in
15 which the hybridization domains specifically hybridize to the target to form a multimeric oligonucleotide/target oligonucleotide aggregate, separating the aggregate from the mixture of oligonucleotides, disassociating the target oligonucleotide from the aggregate, and separating the target oligonucleotide from multimeric oligonucleotide.



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(21) International Application Number: PCT/US97/13010 (22) International Filing Date: 24 July 1997 (24.07.97)		(74) Agent: GREENFIELD, Michael, S.; McDonnell Boehnen Hulbert & Berghoff, 300 South Wacker Drive, Chicago, IL 60606 (US).	
(30) Priority Data: 08/690,300 26 July 1996 (26.07.96)		US	(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).
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(71) Applicant (for all designated States except US): HYBRIDON, INC. [US/US]; One Innovation Drive, Worcester, MA 01605 (US).		(88) Date of publication of the international search report: 16 April 1998 (16.04.98)	
(54) Title: AFFINITY-BASED PURIFICATION OF OLIGONUCLEOTIDES USING SOLUBLE MULTIMERIC OLIGONUCLEOTIDES			
(57) Abstract			
<p>The present invention provides novel compounds and methods for purifying oligonucleotides. The compounds according to the invention are multimeric oligonucleotides comprising a multimerization domain for inducing multimeric oligonucleotide aggregation, a hybridization domain that is complementary to a target oligonucleotide whose isolation is desired, and a linker domain connecting the multimerization domain and the hybridization domain. Other compounds of the invention comprise dendrimers having oligonucleotides with hybridization domains linked thereto. The methods of the invention comprise contacting the compounds of the invention with a solution containing a target oligonucleotide whose purification is desired. The target oligonucleotide hybridizes to the hybridization domain of the inventive compounds, thereby forming an aggregate. Synthetic failure sequences (N-1, N-2, etc.) and other oligonucleotides not complementary to the hybridization domain do not hybridize with the hybridization domain of the compounds and remain free in solution. Conventional size exclusion chromatography or small pore filter membranes are then used to separate the aggregate (and hence target oligonucleotide) from the other oligonucleotides. The aggregate is denatured and the target oligonucleotide separated once again by size exclusion chromatography or with a small pore filter membrane.</p>			

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 97/13010

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 C07H1/06 C07H21/00 C12N15/11

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 IPC 6 C07H C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WOLFE J L ET AL: "MODULATION OF TETRAPLEX FORMATION BY CHEMICAL MODIFICATIONS OF A G4-CONTAINING PHOSPHOROTHIOATE OLIGONUCLEOTIDE" JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, vol. 118, no. 26, 1996, page 6301/6302 XP002041657 see the whole document --- A	1
A	WO 96 11010 A (PHARMAGENICS INC) 18 April 1996 see the whole document --- -/-	1

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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1

Date of the actual completion of the international search

25 February 1998

Date of mailing of the international search report

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INTERNATIONAL SEARCH REPORT

Inte	onal Application No
PCT/US 97/13010	

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHEONG C ET AL: "SOLUTION STRUCTURE OF AN UNUSUALLY STABLE RNA TETRAPLEX CONTAINING G- AND U-QUARTET STRUCTURES" BIOCHEMISTRY, vol. 31, no. 36, 1992, pages 8406-8414, XP002041658 see the whole document ---	1
A	MOHANTY D ET AL: "CONFORMATIONAL POLYMORPHISM IN G-TETRAPLEX STRUCTURES: STRAND REVERSAL BY BASE FLIPOVER OR SUGAR FLIPOVER" NUCLEIC ACIDS RESEARCH, vol. 21, no. 8, 1993, pages 1767-1774, XP002041659 see the whole document ---	1
E	WO 97 48715 A (HYBRIDON, INC.) 24 December 1997 see the whole document -----	1-34

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intel. onal Application No
PCT/US 97/13010

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9611010 A	18-04-96	AU 3636995 A	02-05-96
WO 9748715 A	24-12-97	NONE	

